cultivation methods with BRMM were used, except that various concentrations of the antibiotics were added to the tissue cultures at the time of inoculation. Cultures were harvested after 7 days and the total number of treponemes and the number of motile treponemes were counted. The MICs of penicillin, tetracycline, erythromycin, and spectinomycin were determined to be 0.0005, 0.2, 0.005, and 0.5 μg/ml, respectively. MBC determinations were performed by intradermally injecting samples of each culture fluid into the skin on the shaved backs of rabbits. The injection sites were monitored for 45 days to see if lesions developed. All lesions were examined by dark-field microscopy for motile treponemes to verify an active treponemal infection. The MBCs of penicillin, tetracycline, erythromycin, and spectinomycin were 0.0025, 0.5, 0.005, and 0.5 μg/ml, respectively.

Acknowledgments

I dedicate this article to two scientists who made significant contributions toward the *in vitro* cultivation of *T. pallidum:* Dr. A. Howard Fieldsteel (1918–1982), the director of the laboratory that was first to achieve *in vitro* cultivation of *T. pallidum* in the spring of 1980, and Dr. Tom Fitzgerald (1944–1992), who published several articles beginning in the mid-1970s that characterized and detailed the interaction of *T. pallidum* with several mammalian cell lines. These studies proved critical in understanding the culture conditions necessary to provide an environment conducive for treponemal replication *in vitro*.

[28] Measurement of Invasion by Gentamicin Resistance

By ERIC A. ELSINGHORST

Introduction

The gentamicin survival assay is frequently used for the measurement of eukaryotic cell penetration by bacteria because of its simplicity and sensitivity relative to techniques such as Giemsa staining and direct observation. A search of the literature on bacterial invasion will attest to the growing use and popularity of this assay. The principle of the assay is based on the limited penetration of the aminoglycoside antibiotic gentamicin into eukaryotic cells.[1] Organisms that have penetrated the eukaryotic membrane are intracellular and are therefore protected from the bactericidal effects of gentamicin, whereas extracellular organisms are rapidly

[1] P. Vaudaux and F. A. Waldvogel, *Antimicrob. Agents Chemother.* **16,** 743 (1979).

killed by the antibiotic. After removal of the gentamicin, the eukaryotic cells are lysed to release the intracellular bacteria, which are then enumerated by plate count.[1,2] This methodology has been adapted to measure events that occur subsequent to invasion, such as intracellular survival and replication,[3] as well as intercellular spread.[4] The emphasis of this article is on the bacterial penetration of cultured epithelial cells, but gentamicin also has been used to study bacterial interactions with other eukaryotic cell types, such as macrophages.[5]

The following protocol describes a standard assay that was developed to measure the penetration of the human epithelial cell line Henle 407 by *Salmonella typhi;* however, it has been used successfully for measuring invasion of several different epithelial cell lines by several different enteric pathogens, including *Salmonella* species other than *S. typhi, Shigella* species, and various *Escherichia coli* (enterotoxigenic, enteropathogenic, and enteroinvasive *E. coli*). The protocol can serve only as a guideline as the conditions for optimum invasiveness by a particular pathogen must be empirically determined. There are numerous ways to modify this assay, from the treatment and growth conditions of the eukaryotic and prokaryotic cells to the parameters of the assay. Some of the most useful modifications are described following the standard assay.

Standard Invasion Assay

Day Prior to Assay

Preparation of Epithelial Cell Monolayers. Tissue culture techniques and methodology are not discussed in this article. For a thorough discussion of tissue culture techniques, the reader should consult other references.[6,7] The information given here is intended to present details that are relevant for invasion assays.

A single-cell suspension of the desired epithelial cells is prepared by trypsinization. It is important that a uniform suspension is prepared. Epithelial cell clumps result in an increased background (see Results, below). The suspension is diluted in tissue culture growth medium that is appropriate for the cell line being studied. One milliliter of the diluted cell suspension is added to each well of a 24-well tissue culture plate (1.77

[2] E. Kihlström, *Infect. Immun.* **17,** 290 (1977).
[3] J. A. Devenish and D. A. Schiemann, *Infect. Immun.* **32,** 48 (1981).
[4] E. V. Oaks, M. E. Wingfield, and S. B. Formal, *Infect. Immun.* **48,** 124 (1985).
[5] C. R. Lissner, R. N. Swanson, and A. D. O'Brien, *J. Immunol.* **131,** 3006 (1983).
[6] R. I. Freshney, "Culture of Animal Cells." Alan R. Liss, New York, 1987.
[7] W. B. Jakoby and I. H. Pastan, eds., this series, Vol. 58.

cm^2/well). The number of wells to prepare depends on the number of replicates desired; however, at least three wells should be used for each bacterial strain or specific condition to be studied. The density of the diluted cell suspension is dependent on the cell line being used for the assay. The goal of the dilution is to reach a point such that after overnight incubation, the epithelial cells will be 95 to 100% confluent. The number of cells constituting such a confluent monolayer is dependent on the cell line being used and can range from approximately 7×10^4 to 5×10^5. The number of cells in a monolayer for a specific epithelial cell line can be determined by trypsinization of confluent wells and direct counting by hemocytometer. The epithelial cells are incubated overnight at 37° in a 5% CO_2 humidified atmosphere.

Preparation of Bacterial Cultures. Typically, bacterial cells are grown overnight in LB medium (L-broth)[8] at 37°, shaking at 200 rpm. As discussed below, however, invasion may require specific growth conditions and media. Bacterial cultures should be prepared accordingly.

Day of Assay

Preparation of Epithelial Cell Monolayers. The monolayers can be washed prior to inoculation of the wells, although these washings may not be necessary (see Washes and Inoculation of Monolayers, below). If an antibiotic-containing tissue culture medium is used for growth of the monolayers, the wells must be washed before inoculation. Washings can be performed with antibiotic-free tissue culture medium, balanced salt solution (e.g., Earle's balanced salts), or phosphate-buffered saline (PBS), and are most easily accomplished by aspirating the supernatants from the wells, adding 0.5 to 1.0 ml of washing solution, briefly and gently shaking the plates on a rotating platform, and then repeating the washing procedure. After the final wash, 1.0 ml of balanced salt solution or tissue culture medium is added to each well.

Preparation of Bacterial Cultures. Overnight bacterial cultures are diluted 1:20 to 1:50 into 1 ml of fresh L-broth in 13 × 100-mm glass tubes and grown at 37°, 200 rpm, to midlogarithmic (midlog) phase [$\approx 10^8$ colony-forming units (cfu)/ml]. If bacterial growth conditions or media are other than those described above, the cultures should be passaged so that mid-log-phase organisms are used in the assay.

Assay. A 25-μl aliquot of a mid-log-phase culture is added directly to the tissue culture medium bathing the monolayers. This inoculum contains about 2 to 5×10^6 cfu resulting in a multiplicity of infection (m.o.i.) of

[8] J. H. Miller, "Experiments in Molecular Genetics," p. 433. Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1972.

approximately 10 bacteria per epithelial cell. After inoculation of all wells, the 24-well plate is briefly and gently shaken on a rotating platform to distribute the inoculum throughout the tissue culture medium. If desired or necessary, the plate can be centrifuged to initiate contact between the bacteria and epithelial cells. Centrifugation is typically performed for 5 min at 600 *g* and room temperature in a swinging bucket rotor fitted with microtiter plate adaptors. The 24-well plate is then incubated at 37° for 2 hr in a 5% CO_2 humidified atmosphere. This incubation is referred to as the *invasion incubation.* At the time of inoculation, a quantitative plate count is performed on the mid-log-phase culture to determine the total number of input bacteria.

At the end of the invasion incubation the monolayers are washed three times with balanced salt solution or PBS. The purpose of these washes is to remove bacteria that have not adhered to, or invaded, the epithelial cell monolayer. The washes are performed as described above (Preparation of Epithelial Cell Monolayers). After the final wash, 1.5 ml of tissue culture medium or balanced salt solution containing 100 μg/ml gentamicin is added to the monolayer. The 24-well plates are then incubated at 37° for 2 hr in a 5% CO_2 humidified atmosphere. This incubation is referred to as the *gentamicin-kill incubation.* A larger volume of medium is added to the wells during the gentamicin-kill incubation than during the invasion incubation to ensure that bacteria that may have adhered to the walls of the wells are killed by the antibiotic.

After the gentamicin-kill incubation, the monolayers are washed twice with Earle's balanced salt solution or PBS to remove the gentamicin. After aspiration of the final wash, 1 ml of 0.1% Triton X-100 in deionized water is added to the wells to lyse the monolayers. To aid lysis, the plates are shaken on a rotating platform for 5 min. Liberated bacteria are quantitated by dilution and plating on an appropriate medium. The extent of dilution required to achieve countable numbers depends on the epithelial cells used in the assay and the invasiveness, or lack thereof, of the organism being studied, but can range from 10^0 to 10^{-5}.

Results

The results of an invasion assay are often presented as the percentage of the input number of bacteria that have survived the bactericidal action of gentamicin, i.e., (the total cfu recovered from a well/divided by the CFU in the inoculum) $\times 100$. Performing the experiment in triplicate (i.e., three replicate wells for each organism) allows calculation of a mean and a range for each assay. As discussed below, the assay is variable on a daily basis; therefore, invasion data are frequently reported as the results

of a single experiment. These results should, however, be reflective and consistent with those obtained in replicate experiments.

Invasion assays can be highly variable on a day-to-day basis. This variability may be related to the overall physiological state of both the prokaryotic and eukaryotic cells. Therefore, it is important to include known invasive and "noninvasive" strains as positive and negative controls, respectively. These inclusions are particularly important if attempts are being made to establish invasiveness by a pathogen or by mutant strains. Typical noninvasive bacteria include laboratory strains of *E. coli*, such as HB101,[9] C600,[10] and MC4100,[11] that are used in molecular biological techniques. It must, however, be noted that noninvasive strains can survive the gentamicin-kill incubation, albeit at a low level, thereby establishing the background, or sensitivity limit, of the assay. It is uncertain if survival of noninvasive strains represents actual internalization or the sequestration of organisms in an external environment that is protected from gentamicin or its bactericidal effects (see Limitations of Assay, below). It is, however, important to include a negative control as an internal measure for the sensitivity of the assay on a particular day. Although varible on a daily basis, background recovery from negative controls can typically range from 0.001 to 0.05% depending on the cell line used in the assay.

A problem with calculating invasion as the percentage of input bacteria that have survived gentamicin treatment is the growth of the inoculum during the invasion incubation. Many organisms are capable of replicating in the tissue culture media or salt solutions that are used in the assay. Because of this growth, the final number of bacteria available to invade the monolayer by the end of the invasion incubation will have increased relative to that at the start of the assay. Additionally, the growth rates of various organisms in these media are not identical. To correct for bacterial growth during the invasion incubation, quantitative plate counts can be performed for each organism at the end of the invasion incubation. A method for accomplishing this count is to add Triton X-100 to a final concentration of 0.1% to an infected well. After allowing for lysis of the monolayer, a quantitative plate count will include all bacteria in the well (i.e., bacteria that are not associated with the monolayer, as well as those that have either adhered to or invaded the monolayer). Calculations of percentage invasion using the number of bacteria present in the well at the end of the invasion incubation allow for a closer comparison of the

[9] H. W. Boyer and D. Roulland-Dussoix, *J. Mol. Biol.* **41**, 459 (1969).
[10] R. K. Appleyard, *Genetics* **39**, 440 (1954).
[11] C. A. Kumamoto and J. Beckwith, *J. Bacteriol.* **154**, 253 (1983).

relative invasiveness of different pathogens for a particular epithelial cell line.

Another measure of invasiveness is the invasion index,[12,13] which is the number of invaded organisms taken as a percentage of the number of adhered organisms. To calculate the invasion index, adherence and invasion must be simultaneously determined. For measurement of adherence, a parallel set of wells are treated as described for the invasion assay; however, at the end of the invasion incubation, the wells are washed six times and then lysed with Triton X-100 as described for the invasion assay. As these wells have not been exposed to gentamicin, recovered bacteria represent those that have adhered to or invaded the monolayer. The invasion index is a measure of an organism's ability to adhere to a eukaryotic cell and how likely it is that that interaction will lead to internalization. Adherence is most likely a prerequisite for invasion of epithelial cells by any bacterial pathogen; however, that pathogen may bind to an epithelial cell membrane without subsequent internalization. Therefore, the index is most useful when comparing invasiveness between similar pathogens or isogenic strains or when measuring the capacity of a particular pathogen to invade different cell lines.

Intracellular Replication. The number of organisms recovered from an invasion assay may represent not only those organisms that have invaded the monolayer, but also those that have undergone replication after penetration of the epithelial cell. The extent of intracellular replication is dependent on both the bacterial strain and the cell line being studied in the assay (Fig. 1). For example, *Shigella flexneri* replicates rapidly inside many cell lines; however, intracellular replication of *Salmonella* species is dependent on the cell line examined, with rapid intracellular replication occurring within cells derived from specific tissues or organs, such as kidney. The extent to which intracellular replication contributes to the overall recovery from an invasion assay must be determined for each organism and epithelial cell line studied.

For measurement of bacterial intracellular multiplication, the standard invasion assay is modified by extending the length of the gentamicin-kill incubation over several hours. Separate 24-well plates are prepared for each time point with triplicate wells for each organism to be studied. The plates are infected simultaneously and treated as described for the standard assay. After the invasion incubation, all plates are washed and treated with gentamicin; however, the length of the gentamicin-kill incubation is varied over several time points. Time points can be taken at 1, 3, 6, 10,

[12] G. W. Jones, L. A. Richardson, and D. Uhlman, *J. Gen. Microbiol.* **127**, 351 (1981).
[13] E. A. Elsinghorst and D. J. Kopecko, *Infect. Immun.* **60**, 2409 (1992).

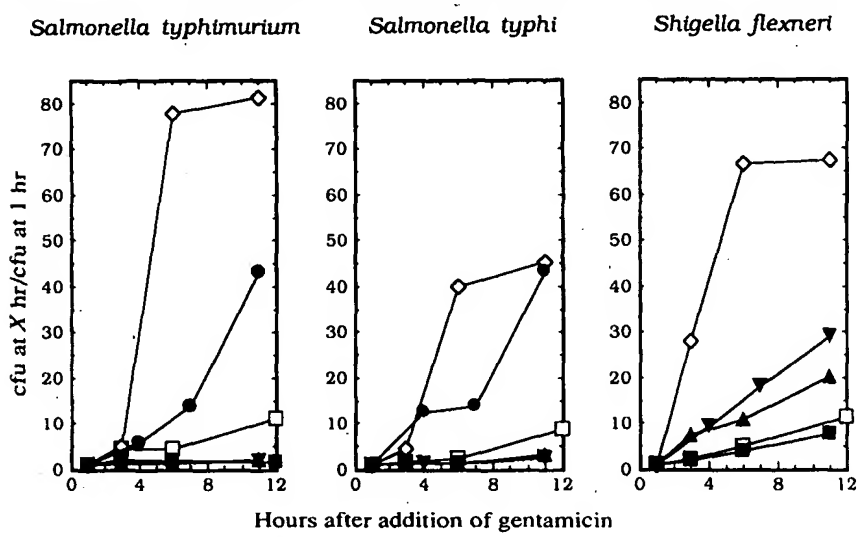

FIG. 1. Intracellular multiplication of enteric pathogens in several different epithelial cell lines. Multiplication assays were performed as described in the text, with the first time point taken after 1 hr exposure to gentamicin. The symbols represent the following cell lines (which are further described in Table I): (◇) A-498, (▲) HCT-116, (■) HCT-8, (▼) HeLa, (●) LLC-MK2, (□) T84.

16, and 24 hr of exposure to gentamicin, but the placement of time points will be determined by the rate of intracellular replication and the effects this replication has on the monolayer. At a time point, a 24-well plate is washed, the monolayers are lysed, and the bacteria are quantitated by plate count. The bacterial recoveries from the various time points are compared with the recovery at the initial time point. As gentamicin kills extracellular bacteria, an increase with time in the recovery of bacterial cells would reflect intracellular growth of the organism.

This methodology cannot distinguish between bacterial turnover within the eukaryotic cell (i.e., concurrent multiplication and death) and static maintenance of bacterial numbers (i.e., no or limited growth without bacterial death). Therefore, if the intracellular number of organisms does not increase with time, the possibility of intracellular replication still exists.

Irrespective of the capacity to replicate, intracellular bacteria can result in toxicity and lysis of the epithelial cells, particularly during the extended incubation times of a multiplication assay. To minimize these problems if they are encountered, the assay can be further modified by decreasing the multiplicity of infection, decreasing the length of the invasion incubation, and decreasing the length of the initial gentamicin time point. Addi-

tionally, as gentamicin may slowly penetrate the eukaryotic membrane, more reproducible replication curves may be obtained by decreasing the gentamicin concentration to 20 μg/ml.

Viability of Monolayer. Epithelial cell attachment and viability may be influenced during the course of an invasion assay by several factors such as extracellular and intracellular bacterial growth, medium acidification, and bacterial production of toxins. Death of epithelial cells or their release from the monolayer decrease bacterial recovery. The relative viability and integrity of the monolayers should be evaluated at the end of an invasion assay for each epithelial cell line and organism examined. If the monolayer is compromised, its stability can be increased by decreasing the m.o.i. and the lengths of the invasion and gentamicin-kill incubations. Epithelial cell viability and monolayer integrity can be measured by methods including trypan blue exclusion,[14] acridine orange staining,[15] crystal violet staining,[16] and direct observation under an inverted microscope.

Assay Modifications

There are numerous ways in which this assay can be modified depending on the specific organism that is being studied and the specific cell lines that are being used as hosts for bacterial invasion. These modifications can occur at virtually every step of the assay and in the preparation of both the prokaryote and eukaryote.

Growth of Epithelial Cell Line

The choice of cell line can be critical for establishing invasiveness by a particular pathogen. The physiological state of that cell line can also have a major influence on the outcome of an invasion assay. These variables are related to the presence and distribution of receptors that are necessary for bacterial invasion.

Intuitively, the cell lines used to establish or study bacterial invasion would come from the host tissue or organ that would be a likely target for this activity. Bacterial entry into epithelial cells could be overlooked if an inappropriate cell line was examined. For example, although *Shigella flexneri* and *Salmonella typhi* is capable of invading many epithelial cell lines, enterotoxigenic *E. coli* is specific for the cell lines it will penetrate

[14] M. K. Patterson, this series, Vol. 58, p. 151.
[15] M. R. Melamed, L. R. Adams, A. Zimring, J. G. Murnick, and K. Mayer, *Am. J. Clin. Pathol.* **57**, 95 (1972).
[16] M. S. Donnenberg, A. Donohue-Rolfe, and G. T. Keusch, *J. Infect. Dis.* **160**, 452 (1989).

TABLE I
INVASION OF EPITHELIAL CELLS BY ENTEROTOXIGENIC *Escherichia coli* H10407 AND *Salmonella typhi* Ty2[a]

Cell line	Source	% Invasion[b]		% Relative invasion[c]
		H10407	Ty2	
HuTu80	Human duodenum	0.03	5.14	0.58
HCT-8	Human ileocecum	0.41	1.57	26.11
HCT-116	Human colon	1.06	1.20	88.33
T84	Human colon	0.25	0.29	86.21
HeLa	Human cervix	<0.01	9.66	0.05
Chang	Human liver	0.05	1.84	2.72
A-498	Human kidney	0.18	0.67	18.40
LLC-MK_2	Monkey kidney	<0.01	1.90	0.26

[a] Reproduced with permission from Elsinghorst and Kopecko.[13]
[b] Standard assays were performed as described in the text; however, the length of the invasion incubation was increased to 3 hr for H10407.
[c] Invasion relative to *S. typhi*, representing 100%.

(Table I).[13] Until the specific eukaryotic receptor(s) recognized by the bacterium is identified, a range of cell lines may be required to characterize the invasive phenotype of an organism.

As described in the above protocol, the epithelial cells are grown as a 95 to 100% confluent monolayer. As such, these monolayers would be nonpolar. In a polarized monolayer, such as the intestinal epithelium, cell surface components are localized to specific plasma membrane domains, such as the apical, lateral, or basal cell surfaces.[17] The distribution of a receptor(s) necessary for bacterial invasion may be affected by this localization, thereby influencing the invasive phenotype of a pathogen. Many cell lines that are capable of polarization are available,[17] and the use of these cell lines as polarized monolayers may represent a more accurate model of the epithelial cell as seen by a bacterium. The use of polarized epithelial cell monolayers is discussed in depth in [30] of this volume.

Growth of Bacterial Inoculum

The expression of factors required for the invasive phenotype may be affected by the growth conditions used for the preparation of the bacterial inoculum. Conditions that have been shown to affect invasiveness include

[17] K. Simons and S. D. Fuller, *Annu. Rev. Cell Biol.* **1**, 243 (1985).

variables such as osmolarity,[18] growth phase,[19,20] oxygen availability,[19,20] and temperature.[21] Numerous other modifications could be considered. Varying conditions or growth media in ways that have been shown to affect the regulation of virulence factors (e.g., iron, calcium, pH)[22,23] represents a logical starting place in the search for conditions that may either allow for or increase invasiveness in a specific pathogen. Growth conditions also may affect the expression of additional adherence factors that are needed for optimal invasiveness. Invasion of bacteria grown as shaken or static liquid cultures, as well as cells grown on solid media, can be examined.

Washes and Inoculation of Monolayers

Washing the monolayers and bacterial cells before starting an assay may be necessary if components of media or metabolic products are inhibitory or toxic to either the eukaryote or prokaryote. These washings may not, however, be necessary for optimal invasion (Table II). Monolayer washing was described in the standard assay procedure. Bacterial cultures can be washed by centrifugation and resuspension to the original volume in fresh growth medium, PBS, or a tissue culture solution identical to that bathing the monolayers. The washed bacteria can then be used to inoculate the monolayers as described in the standard assay.

Alternately, the mid-log-phase bacterial culture can be harvested by centrifugation, then resuspended to a density of 2 to 5 $\times$ 10^6 cfu ml in tissue culture medium or balanced salt solution. If washing by centrifugation results in decreased invasiveness, the mid-log-phase culture can be diluted directly into fresh growth medium to the desired final density. After aspiration of the growth medium from the monolayers, 1 ml of diluted bacteria is added to each well, resulting in an m.o.i. of about 10. The monolayers are then treated as described for the standard assay.

Although the standard assay uses an m.o.i. of about 10 bacteria per epithelial cell, this ratio may need to be changed for optimal invasion by a specific organism or during certain experiments such as intracellular multiplication or invasion inhibition assays. It should be noted that increasing the m.o.i. often results in higher background levels (i.e., increased recovery of invasion negative controls).

[18] J. E. Galan and R. Curtiss, III, *Infect. Immun.* **58**, 1879 (1990).
[19] R. K. Ernst, D. M. Dombroski, and J. M. Merrick, *Infect. Immun.* **58**, 2014 (1990).
[20] C. A. Lee and S. Falkow, *Proc. Natal. Acad. Sci. U.S.A.* **87**, 4304 (1990).
[21] A. T. Maurelli, B. Blackmon, and R. Curtiss, III, *Infect. Immun.* **43**, 195 (1984).
[22] J. F. Miller, J. J. Mekalanos, and S. Falkow, *Science* **243**, 916 (1989).
[23] J. W. Foster, *J. Bacteriol.* **173**, 6896 (1991).

TABLE II
EFFECT OF VARIOUS WASHINGS PRIOR TO INOCULATION OF MONOLAYERS ON HeLa CELL INVASIVENESS OF *Salmonella typhi* Ty2

Bacterial washing[a]	HeLa cell washing[b]	% Invasion
Unwashed	−	12.04 ± 0.35
Unwashed	+	13.72 ± 2.80
I	−	13.37 ± 0.63
I	+	14.40 ± 2.60
II	−	11.79 ± 0.34
II	+	12.60 ± 0.60

[a] Bacteria were grown as described for the standard assay, then split into three samples that were treated as follows: unwashed, bacteria were used directly without additional treatment; I, cells were pelleted by centrifugation (5 min, 3000 *g*) and then resuspended in an equal volume of L-broth; II, cells were pelleted by centrifugation (5 min, 3000 *g*) and then resuspended in an equal volume of HeLa cell tissue culture medium. After the indicated treatment, standard assays were performed. Quantitative plate counts were performed on each sample and used in percentage invasion calculations to correct for loss of bacterial cells during washes.

[b] HeLa cell washings: One hour prior to inoculation, HeLa cells monolayers were either (−) untreated or (+) washed once with HeLa cell tissue culture medium.

Centrifugation

For either mode of inoculation, the plates can be centrifuged as described in the standard assay. The primary purpose of this step is to initiate and synchronize contact between the bacteria and the monolayer, with the assumption that any intimate contact or close association required for bacterial penetration can be provided by centrifugation. Centrifugation may not result in optimal invasion, and in some cases it may be detrimental (Table III); however, percentage invasion by some organisms, such as *Shigella flexneri*, can be increased by this step. Therefore, it must be determined for each organism of interest whether or not to include centrifugation as a routine step in a standard assay. In some experiments centrifugation may be desired to synchronize invasion, such as those to determine the optimal length of the invasion incubation. Additionally, centrifugation

TABLE III
CENTRIFUGATION OF INOCULATED MONOLAYERS VS PERCENTAGE RECOVERY FROM HeLa CELLS[a]

Organism	% Invasion	
	Centrifuged	Not centrifuged
Salmonella typhi Ty2	2.58 ± 0.28	4.45 ± 0.54
Salmonella typhi 101018	4.55 ± 0.78	6.64 ± 1.19

[a] Invasion assays were performed on 24-well plates in parallel. One plate was incubated at room temperature and the second plate was centrifuged as described in the standard assay, after which the plates were treated as described for the standard assay.

can be used in experiments studying the effects of various inhibitors on invasion to obviate the concern that the presence of these compounds may be altering an intimate contact or close association step that is required for invasion but not the invasion step itself.

Length of Invasion Incubation

Several elements contribute to the optimal length of the invasion incubation: Is there a need for induction of invasion factors? What specific interactions are required for internalization to occur (e.g., loose adherence, tight adherence)? What are the availability and abundance of receptors? Also, as penetration of the eukaryotic membrane could be considered an enzymatic reaction, the kinetic parameters and constants of that reaction also contribute to the optimal length of the invasion incubation. In consideration of these factors, the length of the invasion incubation can be increased or decreased from the 2-hr incubation described in the standard assay. The results of these changes vary with the organism (Table IV). In any length of invasion incubation, the effect of the bacteria on the viability of the monolayer as a result of extracellular and intracellular growth of the inoculum, medium acidification, toxicity from the release of cytotoxins, or other factors must be considered. By increasing the length of the invasion incubation any negative effects become more pronounced. The viability and attachment of the monolayer should be assessed at the end of the assay.

Gentamicin-Kill Incubation

The gentamicin-kill incubation can be modified by changing the antibiotic concentration and length of the incubation period. These variables

TABLE IV
LENGTH OF INVASION INCUBATION VS PERCENTAGE RECOVERY FROM HeLa CELLS[a]

Organism	Length of invasion incubation (min)	% Invasion
Salmonella typhi Ty2	15	1.85 ± 0.13
	30	2.71 ± 0.54
	60	9.37 ± 0.91
	120	10.24 ± 0.45
Salmonella typhi 101018	15	1.34 ± 0.32
	30	1.26 ± 0.09
	60	4.31 ± 0.11
	120	6.01 ± 0.41
Salmonella typhi I 14	15	1.06 ± 0.12
	30	1.41 ± 0.14
	60	3.51 ± 0.28
	120	3.60 ± 0.62
Salmonella typhimurium C5	15	0.61 ± 0.23
	30	1.43 ± 0.27
	60	1.63 ± 0.22
	120	1.97 ± 0.26
Shigella flexneri M90TW	15	0.32 ± 0.04
	30	0.51 ± 0.03
	60	1.90 ± 0.30
	120	3.11 ± 0.91

[a] The standard invasion assay was modified by altering the length of the invasion incubation. Preparation of bacterial cultures, monolayer inoculation and washings, and gentamicin-kill incubations were performed as described for the standard assay. The total number of bacteria per well was determined at the end of each time point as described under Results to correct for growth of the inoculum during the experiment. These numbers were used in calculating the percentage invasion for each organism and time point, respectively.

are dependent on the sensitivity of the test organism to gentamicin and the type of assay being performed. The concentration of gentamicin should at least equal the minimum bactericidal concentration (MBC) for the organism being studied. The MBC varies depending on the growth medium in which it is determined. It should therefore, be measured in the medium used during the invasion incubation.

Because gentamicin may slowly penetrate the eukaryotic membrane,[1] the antibiotic concentration and incubation times can be decreased if there is concern that gentamicin-mediated death of intracellular bacteria is occurring. The influence of gentamicin leakage on percentage invasion

may, however, be negligible. The gentamicin MBC for *Salmonella typhi* Ty2 is 0.25 to 1.56 μg/ml depending on the growth medium used for the determination. The invasion of HeLa cells by Ty2 was measured in standard assays that were modified by performing the gentamicin-kill incubations with media containing either 5, 20, or 100 μg–ml gentamicin. The percentage invasion of Ty2 was 3.46 ± 0.38, 3.76 ± 0.23, and 3.55 ± 0.89, respectively. Decreasing the length of the gentamicin-kill incubation from 2 to 1 hr did not have an effect on the percentage invasion of HeLa cells by Ty2 in an otherwise standard assay. Gentamicin-mediated intracellular death of bacteria is most likely to be encountered during prolonged incubations, such as those found in intracellular multiplication and intercellular spread assays. For these types of experiments, it is useful to decrease the gentamicin concentration to 20 μg/ml. When altering the parameters of the gentamicin-kill incubation, bear in mind that the length of the incubation at the selected drug concentration must be sufficiently long to ensure death of extracellular bacteria.

Lysis of Monolayer

Intracellular bacteria surviving the gentamicin treatment are released by lysing the epithelial cells with a detergent; however, as these detergents also can lyse bacteria, it is important to evaluate bacterial viability under the conditions used to lyse the monolayers (i.e., type and concentration of detergent and length of incubation). Bacterial viability can be measured by diluting a mid-log-phase culture in lysis solution, incubating for the prescribed length of time, and then plating on appropriate growth media. Recovery of cells diluted in detergent should be compared with recovery of cells diluted in water. PBS can be substituted for water in lysis solutions if the dilution of bacteria in water decreases cell viability. Bacteria that have penetrated the epithelial cell may be physiologically stressed and more susceptible to detergents than a mid-log-phase culture. Therefore, the epithelial cell lysis conditions resulting in the maximal recovery of bacteria may need to be determined empirically.

The ease of eukaryotic cell lysis varies with cell line and degree of polarization. Some cell lines are difficult to lyse and may require physical assistance (such as repeated pipetting of the lysate) to achieve disruption of the monolayer. The extent of lysis can be examined by direct observation with an inverted microscope. The type and concentration of detergent, and length of incubation can be altered depending on the sensitivity of the cell line. Detergents that can be used for lysis include Triton X-100 and sodium deoxycholate at concentrations ranging from 1.0 to 0.01% in distilled water. The length of the lysis incubation can be increased from

5 min depending on the sensitivity of the eukaryote and prokaryote to the concentration of detergent used for lysis.

Limitations of Assay

Two major questions regarding the limitations of the gentamicin survival assay arise from the principles on which it is based: that there is limited penetration of gentamicin into eukaryotic cells, and that only organisms that are removed from the external environment (i.e., having penetrated the eukaryotic cell) are protected from the bactericidal effects of the antibiotic. These two questions are: Does gentamicin kill any intracellular organisms? and Does gentamicin kill all extracellular organisms?

Does gentamicin kill any intracellular organisms? Vaudaux and Waldvogel[1] studied the penetration of gentamicin into human polymorphonuclear leukocytes and showed that these cells did not accumulate the antibiotic while resting or during active phagocytosis; however, their gentamicin binding studies did not preclude slow penetration of the antibiotic through the eukaryotic membrane. If gentamicin is entering the epithelial cells during the course of an invasion assay, is it reaching concentrations sufficient to decrease bacterial viability? The answer to this question depends partly on the rate at which gentamicin penetrates the eukaryotic membrane and the sensitivity of the test organism to the antibiotic. As described earlier, varying the concentration of the antibiotic or length of the gentamicin-kill incubation has little or no effect on the percentage invasion of HeLa cells by *Salmonella typhi,* suggesting that in this specific example, gentamicin-mediated intracellular killing is not occurring; however, gentamicin might permeate sufficiently to decrease bacterial viability during prolonged gentamicin-kill incubations, such as those used during intracellular multiplication assays. The effect of gentamicin on intracellular bacterial populations needs to be examined on a case-by-case basis.

Does gentamicin kill all extracellular organisms? This question can be asked in the following way: Is there an extracellular environment that is sequestered from gentamicin or one that prevents gentamicin from exerting its bactericidal effects? Although it is not known if there are extracellular environments that can exclude gentamicin, there are conditions that are known to reduce its efficacy. Gentamicin is an aminoglycoside antibiotic, and initial uptake of these antibiotics by bacteria is strongly influenced by bacterial membrane potential and requires active protein synthesis.[24] Aminoglycosides are less effective at low pH[25] or under anaerobic condi-

[24] B. D. Davis, *Microbiol. Rev.* **51,** 341 (1987).
[25] M. Barber and P. M. Waterworth, *Br. Med. J.* **1,** 203 (1966).

tions (due to depressed bacterial membrane potential).[24,26] Extracellular microenvironments that reduce the efficacy of the drug and/or reduce bacterial uptake could theoretically diminish the effective concentration of the antibiotic to a level below that required to kill bacteria. These microenvironments could be formed at eukaryotic cell surfaces by folds or ruffles of the cell membrane or in intracellular spaces. Bacteria contained within such microenvironments could potentially survive gentamicin treatment and, following lysis of the eukaryotic cell, be counted as invasive. This extracellular protection may account for the recovery of "noninvasive" controls from invasion assays.

Recovery of noninvasive controls illustrates the need to confirm the invasive phenotype by other methods. This confirmation is particularly important when identifying and characterizing invasion by a pathogen for which the invasive phenotype has not been described previously, or if the percentage recovery of an organism is low relative to the control strains. Electron microscopy has frequently been used to verify bacterial penetration of eukaryotic cells. Measurement of invasion by alternate methods is discussed in depth in [29] of this volume.

[26] M. Kogut, J. Lightbown, and P. Isaacson, *J. Gen. Microbiol.* **39**, 155 (1965).

[29] Measurements of Invasion by Antibody Labeling and Electron Microscopy

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Introduction

Many bacterial pathogens have the capability to enter and reside within eukaryotic cells.[1-3] As described in [28], various methodologies have been developed to assess bacterial invasion into host cells *in vitro*, using infection models in combination with microscopy, metabolic labeling assays, or measurement of the intracellular survival of the bacteria. The purpose of this article is to outline and discuss practical strategies and technical details for successful immunomicroscopic measurement of bacterial inva-

[1] B. B. Finlay and S. Falkow, *Microbiol. Rev.* **53**, 210 (1989).
[2] M. J. Wick, J. L. Madara, B. N. Fields, and S. J. Normark, *Cell (Cambridge, Mass.)* **67**, 651 (1991).
[3] S. Falkow, *Cell (Cambridge, Mass.)* **65**, 1099 (1991).